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## Functional analysis of circular and linear bacteriocins of Gram-positive bacteria

Kemperman, Robèr Antoine

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# Chapter 2

## **A Salt-Inducible Expression System for Production of Pediocin PA-1 Mutants.**

Rober A. Kemperman, Ronald Langstraat, Oscar P. Kuipers and Jan Kok.

## ABSTRACT

**We describe an effective inducible gene expression system for the production of pediocin mutants with altered bactericidal activities. This was achieved by uncoupling the expression of the pediocin structural gene from the secretion machinery by use of a twin-plasmid system. By expressing the genes of the secretion machinery (encoded by the *pedC* and *pedD* genes) under regulation of a NaCl-inducible promoter, the chance of isolating false-negative mutants with mutations in *pedCD* was reduced. A number of position-randomized pediocin PA-1 mutants with altered bactericidal activities were produced with this expression system, showing its effectiveness. Replacing the alanine residue at position 34 in pediocin PA-1 by a positively or negatively charged residue strongly reduced bacteriocin activity. Changing the aspartic acid residue at position 17 alters target cell specificity.**

## INTRODUCTION

Many lactic acid bacteria produce small, ribosomally synthesized antimicrobial peptides called bacteriocins (19, 24). Bacteriocins are membrane permeabilizing cationic peptides of usually less than 50 amino acid residues (24). Bacteriocins may be divided into five classes (23, 31, 33), of which the best characterized are the Class I bacteriocins or lantibiotics, containing modified residues, and the Class II bacteriocins lacking these modifications (24, 31).

One of the most thoroughly investigated members of the Class II bacteriocins is pediocin PA-1 produced by *Pediococcus acidilactici* (15, 42). The operon involved in pediocin PA-1 production consists of four genes. The *pedA* gene encodes the actual bacteriocin, *pedB* encodes immunity, while *pedC* and *pedD* specify proteins involved in the secretion and processing of the bacteriocin (42). PedD contains the active site domain responsible for cleavage of the double-glycine leader of pediocin PA-1 (42). The function of PedC is still unclear but it is essential for bacteriocin production (42).

Pediocin PA-1 is a member of the type-IIa sub-group of bacteriocins. Typical for bacteriocins of this class are their high overall sequence identity, the conserved sequence motif (YYGN~~GV~~XCXKXXCXVD/NWG/A) in the N-terminal part of the molecule (32) and a variable C-terminal region. Within this group of bacteriocins, a distinction can be made between those that contain a single disulfide bridge between the cysteines in the conserved sequence, like sakacin P, sakacin A, leucocin A and mesentericin Y105 (11, 14, 16, 40), and those that have a second disulfide bridge in the C-terminus, like pediocin PA-1, coagulins, enterocin A and divercin V41 (2, 15, 25,

28). In general, the latter bacteriocins have a wider antibacterial spectrum and are more potent than those with only one disulfide bridge (7, 9). The second disulfide bridge in pediocin PA-1 was found to be very important, but not essential, for bacteriocin activity (7, 9, 30).

A domain swapping study showed that the C-terminal region of pediocin PA-1 is most important with respect to target cell specificity and that total activity is also dependent on the specific N-terminal sequence (8). The importance of the overall amino acid sequence of the bacteriocin is also reflected by substitutions created in the primary bacteriocin sequence: most of these resulted in a severely reduced activity of the peptide (30).

Heterologous expression has been achieved for several Class IIa bacteriocins (36). In most cases, the native biosynthetic genes are expressed either with or without host-specific promoters, as has been done for pediocin PA-1 (27, 42). Alternatively, leader peptides have been exchanged or heterologous ABC-transport and processing systems have been directly used to achieve bacteriocin production (1, 18). In a minority of cases bacteriocins have been heterologously secreted using the general secretion pathway as, for instance, has been done for pediocin PA-1 (29) and mesentericin Y105 (5).

In this article we describe the uncoupling of the expression in *Lactococcus lactis* of the pediocin structural gene from that of the genes of the secretion machinery (*pedC* and *pedD*) by using a twin-plasmid system and an NaCl-inducible promoter of lactococcal origin,  $P_{gad}$  (38). The usefulness of this system is demonstrated by performing a fast screen in *L. lactis* of pediocin mutants with altered bactericidal activities and target cell specificities.

## MATERIALS AND METHODS

### Bacterial strains, plasmids and media

The strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* was grown at 30°C in two-fold diluted M17 broth ( $\frac{1}{2}$ M17; Difco, West Molesey, United Kingdom) containing 1.9%  $\beta$ -glycerophosphate (Sigma Chemical Co., St. Louis, Mo.) or on  $\frac{1}{2}$ M17 agar plates containing 1.5% agar (Benton, Dickenson and Company, LePont de Claix, France). Both media were supplemented with 0.5% glucose (G $\frac{1}{2}$ M17). Chloramphenicol and erythromycin (both from Sigma Chemical Co., St. Louis, Mo.) were used at final concentrations of 5  $\mu$ g/ml unless indicated otherwise. When combined 2.5  $\mu$ g/ml of each antibiotic was used. *Lactobacillus saké* was grown in De Man Rogosa and Sharpe (MRS; Merck, Darmstadt, Germany) broth at 30°C unless mentioned otherwise; erythromycin and chloramphenicol were used together at 2.5  $\mu$ g/ml each. *Listeria innocua* was grown in G $\frac{1}{2}$ M17 at 37°C, while *Pediococcus pentosaceus* PPE1.2, *P. pentosaceus* FBB and *Enterococcus faecalis* 1 were grown in MRS at 37°C.

**Table 1** Bacterial strains and plasmids

Strain or plasmid	Description*	Reference
<b>Strains</b>		
<i>L. lactis</i> MG1363	Plasmid free derivative of NZDO712	(12)
<i>L. lactis</i> LL302	MG1363 derivative carrying a single copy of <i>repA</i> in <i>pepX</i>	(26)
<i>P. pentosaceus</i> PPE 1.2	Pediocin-sensitive indicator	(34)
<i>Lb. sake</i> ATCC 15521	Pediocin-sensitive indicator	Lab collection
<i>Lis. innocua</i> BL86/26	Pediocin-sensitive indicator	Lab collection
<i>E. faecalis</i> 1	Pediocin-sensitive indicator	Lab collection
<b>Plasmids</b>		
pMC117	Em <sup>r</sup> , carrying <i>ped</i> operon	(6)
pMG36e	Em <sup>r</sup>	(41)
pMG36c	Cm <sup>r</sup> , derivative of pMG36e	Lab collection; unpublished
pRK118	Cm <sup>r</sup> , <i>ped</i> operon from pMC117 in pMG36c	this work
pRK119	Cm <sup>r</sup> , deletion of Km <sup>r</sup> from pRK118	this work
pRK130	Cm <sup>r</sup> , derivative of pRK119 lacking <i>pedC</i>	this work
pABC1	Cm <sup>r</sup> , derivative of pRK119 lacking <i>pedD</i>	this work
pABC2	Cm <sup>r</sup> , derivative of pRK119 lacking <i>pedD</i>	this work
pHBK07	Em <sup>r</sup> , <i>pedC</i> and <i>pedD</i> downstream of P <sub>32</sub>	H Karsens; unpublished
pNS3Z	Em <sup>r</sup> , Ori <sup>r</sup> , Rep <sup>r</sup> , <i>lacZ</i> downstream of P <sub>gad</sub>	(38)
pNS3CD	Em <sup>r</sup> , Ori <sup>r</sup> , Rep <sup>r</sup> , <i>pedC</i> and <i>pedD</i> cloned in pNS3Z	this work

\*Cm<sup>r</sup>, Chloramphenicol resistance Em<sup>r</sup>, Erythromycin resistance Km<sup>r</sup>, Kanamycin resistance,

## General methods and materials

Molecular cloning techniques were performed essentially as described by Sambrook et al. (37). Restriction enzymes, T4-DNA ligase and Expand DNA-polymerase were obtained from Roche (Roche Diagnostics GmbH, Mannheim, Germany) and used as specified by the supplier. Plasmids were introduced in *L. lactis* by electro-transformation as described by Holo and Nes (17) using glycine at a concentration of 1% (w/v).

## Sequencing

To obtain templates for sequencing, PCR reactions were performed in quadruplicate using primers Cm-For and pedA-T7. After mixing, the PCR product was purified using the PCR-purification kit (Roche Diagnostics GmbH). Sequencing primers Cy5-P32.uni and Cy5-T7 anneal to this PCR-product. Sequencing was performed using the ALFII-system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) according to the protocols of the supplier, with the following modifications: the power for the long-read gels was set at 15W, the power for the high-resolution gels was set at 18W.

## Plasmid constructions

Unlike previously reported (6) pMC117 was determined to be constructed by ligating the plasmids pMC116 I and pMG36e, both digested by *Sma*I. Of the possible ligation products, the one in which the P<sub>32</sub> promoter drives the expression of the *ped* operon was selected. To obtain the operon on a plasmid carrying a chloramphenicol resistance gene, pMC117 was digested with *Kpn*I and *Sac*I. This fragment was cloned into pMG36c digested with *Kpn*I and *Sac*I, resulting in pRK118. A derivative carrying a deletion of the kanamycin

resistance gene was constructed by digestion of pRK118 with *Bgl*II and *Bam*HI followed by selfligation, resulting in pRK119.

Two stable and efficient plasmid-based systems for producing mutant pediocins were obtained by deleting *pedD* through restriction of pRK119 with *Mva*I, followed by selfligation (pABC1) or by restriction with *Pvu*II and *Swa*I, followed by selfligation (pABC2). In pABC1, contrary to pABC2, part of *pedD* can still be expressed resulting in an N-terminal truncated protein. The genes for the pediocin secretion machinery were cloned behind a NaCl-inducible promoter by digesting pHBK07 (unpublished), a plasmid harboring *pedC* and *pedD* behind P<sub>32</sub>, with *Kpn*I and *Sac*I and ligating the fragment containing *pedC* and *pedD* in pNS3Z, containing the NaCl-inducible *gadC* promoter P<sub>gad</sub> (38), cut with *Kpn*I and *Sac*I.

### Randomization of amino acids at specific positions in pediocin PA-1

Mutations in *pedA* were created using the mega-primer method (3). The primers used in this study are listed in Table 2. In the first round of PCR the primer pedA-F1 and a primer with a randomized codon were used. The resulting PCR product was used as a primer for the second round of PCR with the primer pedAr1. The PCR products were restricted with *Xho*I and *Xba*I and ligated into pABC2 digested with *Sal*I and *Nhe*I. The ligation mixtures were digested with *Sal*I to select for plasmids containing a PCR product. Transformants were examined for the presence of a mutated plasmid by screening for the introduced *Eco*RV site.

Mutations in *pedA* in the codons for cysteine 44, histidine 42 and glycine 40 were introduced in a single PCR with the primers pedAf1 and pedA-Cys44, pedA-His42 and pedA-Gly40, respectively, the products of

**Table 2** Primers used in this study\*

Name	sequence
pedA-F1	5'-CGC <u>CTCGAG</u> TTAAAGAAG <i>Sal</i> I
pedA-R1	5'-GCG <u>TCTAGATATC</u> GCTAGCATTTATGATTACCTTG <i>Xba</i> I <i>Eco</i> RV
pedA-T7	5'- <u>AAC</u> TAAATACGACTCACTATAGGG CCTAGTAAATAAGTCCAAAGC T7-sequencing tag
Cm-For	5'-GAGATAATGCCGACTGTAC
Cy5-P32.uni	5'-CGGAGGAATTTTCAAATGGC
Cy5-T7	5'-TAATACGACTCACTATAGGG
Mutational primers	
pedA-Gly10	5'-CCAGTCAACAGAGCAGGAATGTTT <b>NNN</b> ACAAGTAACC
pedA-Ser13	5'-CCAGTCAACAGAGC <b>NNN</b> ATGTTTGC
pedA-Asp17	5'-CCTTACCC <b>CANNNA</b> ACAGAGC
pedA-Trp18	5'-GGTAGCCTTACC <b>NNNG</b> TCAACAGAGC
pedA-Cys24	5'-CATTATTGATTAT <b>NNN</b> AGTGGTAGC
pedA-Ala34	5'-CCACCA <b>GTNNN</b> CCATGCCATAGC
pedA-Gly40	5'-GCGT <u>TCTAGAG</u> GCTAGCATTTATGATT <b>NNNT</b> TGATGTCCACC <i>Xba</i> I
pedA-His42	5'-GCGT <u>TCTAGAG</u> GCTAGCATTT <b>NNN</b> ATTACCTTG <i>Xba</i> I
pedA-Cys44	5'-GCG <u>TCTAGA</u> CGTAGCT <b>NNNT</b> TTATGATTACCTTG <i>Xba</i> I

\* Introduced restriction sites and 5' tags used for sequencing are underlined. Mismatches in the primers are indicated in bold. **NNN** indicates a randomized codon; the name of the primer reflects the amino acid and its position in mature pediocin PA-1 of which the codon was randomized.

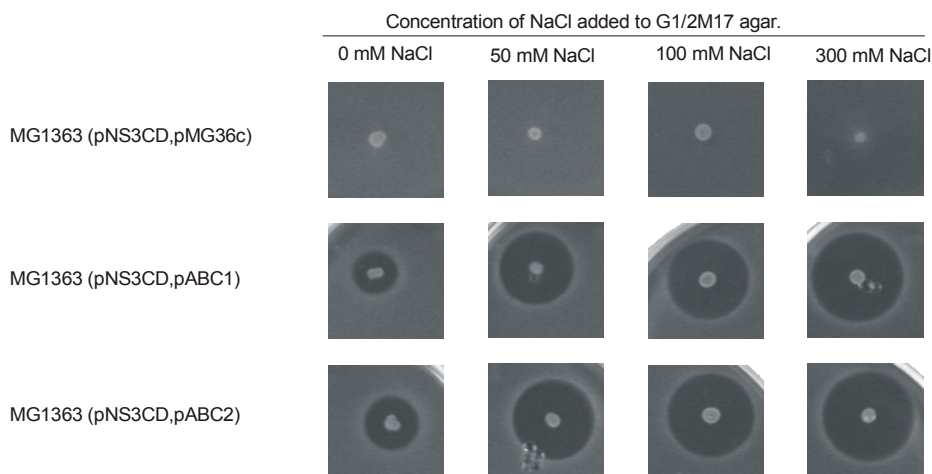


Figure 1: NaCl-induced pediocin PA-1 expression in *L. lactis* MG1363.

Overnight cultures of *L. lactis* MG1363 (pNS3CD) with, in addition, pABC1, pABC2 or pMG36c were spotted on G $\frac{1}{2}$ M17 plates containing increasing amounts of added NaCl. After overnight incubation at 30°C pediocin PA-1 production was determined in a colony overlay assay using *Lb. saké* as indicator.

which were similarly ligated into pABC2. Transformants were selected by screening for absence of the *SalI* site in the resulting vectors. Transformants were screened for activity by plate assays. The *pedA* genes on plasmids of selected clones were subjected to nucleotide sequencing to determine the mutation they contained.

### Bacteriocin assays

Bacteriocin was produced in broth or on plates containing 300 mM NaCl for induction of  $P_{gad}$ . Screening of *L. lactis* transformants for pediocin production was performed by growing the cells overnight (ON) in a microtiterplate. The cell cultures were spotted on G $\frac{1}{2}$ M17 plates containing 300 mM NaCl. After ON growth the cells in the colonies were killed by exposure to chloroform vapor for 15 min. After 30 min exposure to air, the colonies were covered with a top agar layer of the medium used to grow the indicator strain and containing a 100-10000 fold dilution of said indicator grown ON. After ON incubation the plates were examined for halo's of growth inhibition. To compare bacteriocin activities, producing strains were grown for 22 hours in G $\frac{1}{2}$ M17 broth with antibiotics and 300 mM NaCl. Subsequently, bacteriocin activity was determined in a critical dilution-assay as described previously (13) using 200  $\mu$ l final volume in a microtiterplate.

TABLE 3: Effect of amino acid substitutions in pediocin PA-1 on bacteriocin activity<sup>1</sup> against different indicators

Mutation	<i>Lb. saké</i>	<i>Lis. innocua</i>
wt	++	++
A34A	++	++
A34M	++	++
A34V	++	++
A34F	++	++
A34T	++	++
A34I	++	++
A34H	++	++
A34G	+	+
A34W	+	+
A34Y	+	+
A34D	+/- <sup>2</sup>	-
A34R	-	-
A34P	-	-
A34stop	-	-
S13H	++	++
S13R	++	++
S13G	++	++
S13P	+/-	+
S13T	++	++
D17V	+/-	+
D17L	+/-	+
D17F	+/-	+/-
G10V	++	++
G10Y	++	++
G10R	++	++
G40Y	-	-
G40D	-	-
G40R	-	-
G40A	+/-	+
G40L	+	+
G40T	+/-	+
G40P	+	+
W18F	-	-

<sup>1</sup> activity was determined in a colony overlayer assay after ON production on G $\frac{1}{2}$ M17 plates with 300mM NaCl

<sup>2</sup> activity was shown after prolonged incubation 48 hours at 37°C

(-) no activity.(+/-) low activity, halo 1-10mm; (+) medium activity, halo 10-20mm. (++) high activity, halo >20mm (wildtype)

Indicators used were *Lb. saké* grown in MRS at 30°C used at a 10<sup>-2</sup> dilution and *Lis. innocua* grown in G $\frac{1}{2}$ M17 at 30°C, used at a dilutions of 10<sup>-4</sup>.



## RESULTS

### Uncoupling of pediocin PA-1 expression and secretion.

Initially, randomization of specific codons in *pedA* was performed using pMC117 (42). This proved to be very difficult due to instability of the plasmid. Also, expression of *pedC* and *pedD* under control of the constitutive lactococcal  $P_{32}$  promoter, in the absence of a secretable protein, caused plasmid instability (data not shown). As we suspected that this was caused by overexpression of the secretion and processing genes, which specify a membrane-located transporter with a protease function, we decided to uncouple the expression of *pedAB*, encoding pediocin PA-1 and its cognate immunity protein, from *pedCD*. The genes *pedC* and *pedD* were successfully cloned behind the inducible promoter  $P_{gad}$ , creating pNS3CD. Instability of this plasmid was not observed even when NaCl concentrations of up to at least 300 mM were used (data not shown).

Combining pNS3CD with pABC1 or pABC2 yielded a NaCl-inducible system for pediocin expression (Fig. 1). Increasing the concentration of NaCl results in increased pediocin PA-1 production. The pediocin PA-1 expression levels at 300 mM NaCl of *L. lactis* MG1363 (pNS3CD; pABC2) and *L. lactis* MG1363 (pRK119) are both slightly lower than the level of production of the bacteriocin in *P. pentosaceus* PPE1.2 (pMC117). Some expression was still observed in the absence of NaCl; this is likely caused by the NaCl present in the 1/2M17 broth (39). Since the highest level of expression of pediocin PA-1 was observed in the strain with pNS3CD and pABC2, we chose to make mutations in *pedA* in plasmid pABC2.

### Pediocin PA-1 mutants.

To validate the inducible bacteriocin production system, mutations were introduced at several positions in pediocin PA-1 (Table 3). Mutants were identified on the basis of the presence of an additional *EcoRV* site or the loss of the *SalI* restriction site in pABC2. Most of the sequenced *pedA* mutants specified bacteriocins with reduced activity compared to wildtype pediocin PA-1. The consequences for bacteriocin activity of individual mutations is dealt with below.

For the cysteines at positions 24 (C24) and 44 (C44) of mature pediocin PA-1 no active mutants were found. To analyze the effect of substituting the alanine at position 34 (A34), a total of 55 transformants carrying a mutagenized *pedA* fragment in plasmid pABC2 were examined. Of these strains about 20% had lost their activity all together (data not shown). Of the transformants with altered or zero activity levels twenty-nine were sequenced to identify which mutation had occurred (Table 3). Of all possible amino acids at position 34 in pediocin PA-1, thirteen were represented, including tryptophan (once) and methionine (3 times), which are coded for by a single codon. A

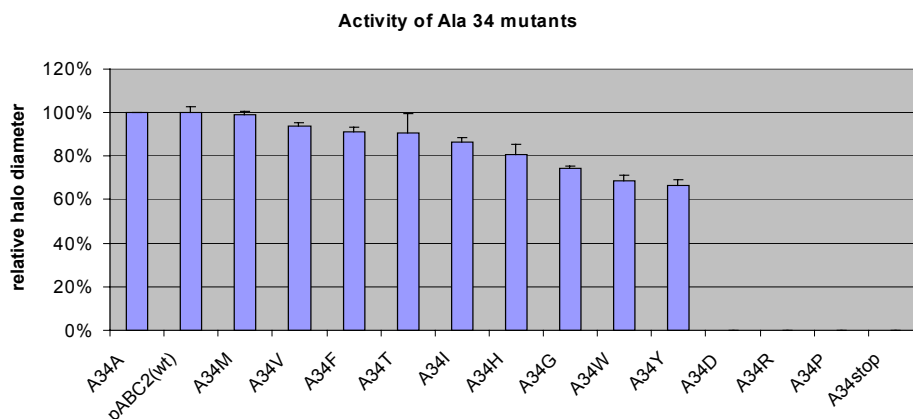


Figure 2: Activity of pediocin PA-1 with substitutions at the position of alanine 34 (Ala34). For each mutant equal amounts of an overnight culture were spotted on a G $\frac{1}{2}$ M17 plate with 300 mM NaCl and grown overnight. The activity was determined in four-fold in a colony overlayer assay using *Lb. saké* as indicator strain. Activity was expressed as the relative halo diameter compared to the control *L. lactis* LL302 (pMGA34A).

mutant carrying a stop codon was also obtained in this way. The activity of the mutant bacteriocins relative to wildtype pediocin PA-1 was determined with an overlayer of the indicator *Lb. saké* on colonies producing the altered bacteriocins. When a hydrophobic or polar residue is present at position 34 (A34V, A34I, A34F, A34G, A34W, A34T) some activity is still retained by the bacteriocins. Activity is lost when the charged residues Arg or Asp (A34R, A34D) are present at this position (Fig. 2). Some activity can still be measured for the A34D mutant of pediocin PA-1 (PedA34D) after production in liquid medium (data not shown) or after prolonged incubation of the producing strain on plates prior to the overlayer assay. The PedA34R mutant shows no activity in both assays. Substituting alanine 34 by proline also caused complete loss of bacteriocin activity, while a stop codon at the position of the A34 codon, as expected, did not lead to active pediocin PA-1 production.

The serine at position 13 (S13) could be replaced by the charged residues arginine or histidine, as well as by the small amino acids glycine, alanine or threonine without much effect on bacteriocin activity. Also, a proline at this position did not cause complete loss of the activity, in contrast to what was observed at position 34. For the glycine residue at position 10 (G10) no drastic effects were observed when it was changed to either a valine, tyrosine or arginine residue. Additional mutations of *pedA* causing complete or incomplete loss of pediocin PA-1 activity were also identified. The substitutions at positions 40 (G40) and 18 (T18), G40Y, G40D, G40R or T18F led to a complete loss of bacteriocin activity while the substitutions G40A, G40L, G40T or G40P in pediocin PA-1 resulted in the production of bacteriocins with decreased

activity (Table 3). Mutants of pediocin PA-1 that showed a shift in activity towards different indicator strains were identified as mutations at position 17 (D17). Three types of mutations exhibit this phenomenon, D17V (isolated 3 times independently); D17L (2 times) and D17F (once). In all cases, activity against *P. pentosaceus* PPE1.2 and *P. pentosaceus* FBB was totally lost. Activity against *Lb. saké*, *Lis. innocua* and *E. faecalis* 1 was reduced to half or less of that of wildtype pediocin PA-1 but the reduction in relative activity was more severe for the *Lb. saké* indicator (Fig 3).

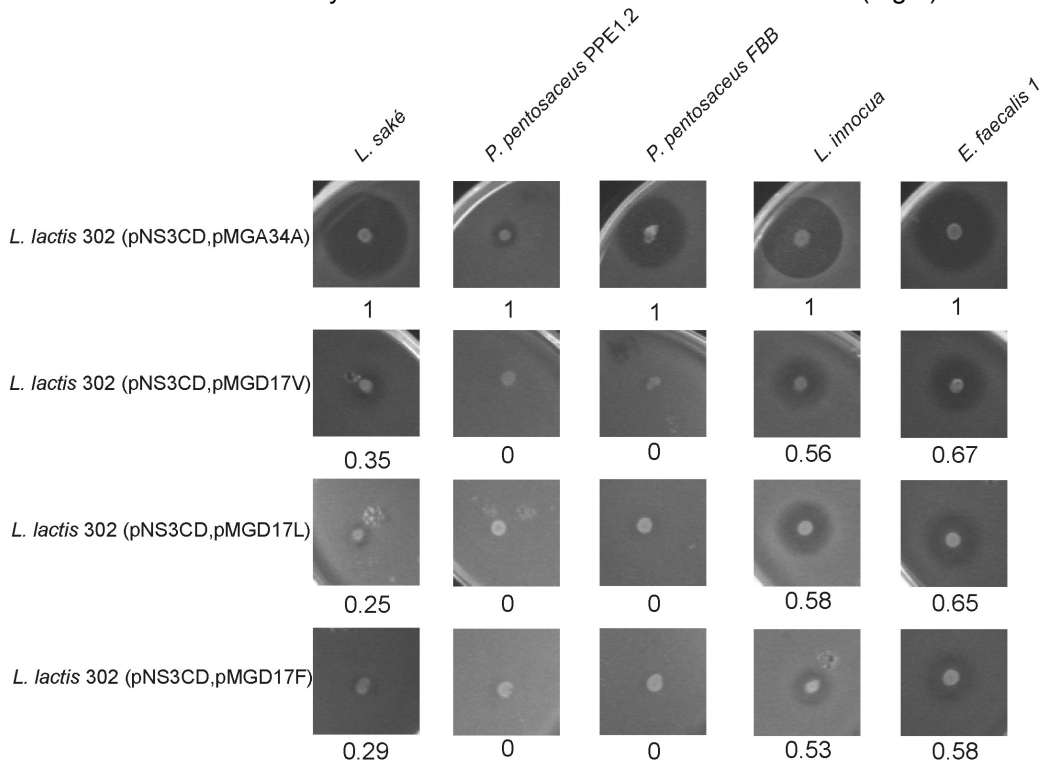


Figure 3: Activity of aspartic acid (D17) mutants of pediocin PA-1. Colonies producing different pediocin PA-1 mutants were spotted on G $\frac{1}{2}$ M17 plates with 300mM NaCl. After ON incubation the activity against different indicators was determined in a colony overlay assay. The indicators were grown in MRS at 37°C (*Lb. saké* was grown at 30°C), *Lis. innocua* was grown in G $\frac{1}{2}$ M17 at 37°C. The numbers below the pictures represent the relative activity, expressed as the measured diameter divided by the diameter of the control halo produced by *L. lactis* 302 (pNS3CD, pMGA34A).

## DISCUSSION

A low-cost salt-inducible system for bacteriocin expression was established in *L. lactis*. In the case reported here the pediocin structural gene and two secretion genes were present on 2 different plasmids. The residual production of pediocin PA-1 by the strains carrying pNS3CD, expressing the secretion proteins, in combination with pABC1 or

pABC2 (specifying the bacteriocin) in the absence of NaCl is probably due to expression from the NaCl-inducible promoter  $P_{gad}$  in  $\frac{1}{2}$ M17 medium, which contains traces of sodium chloride (39). This background level of *pedC* and *pedD* expression does not lead to plasmid instability, in contrast to what was observed when *pedC* and *pedD* were constitutively expressed behind the strong lactococcal  $P_{32}$  promoter. The expression level of pediocin PA-1 using the double-plasmid system is slightly lower than when using a single-plasmid, one-operon system. The lower expression could be due to a combined effect of reduction in gene copies, as a result of the use of two plasmids based on the same replicon, and the lower expression level achieved by the salt-inducible  $P_{gad}$  promoter compared to the  $P_{32}$  promoter (39).

Using this system, the expression level of bacteriocins can be modulated to fit specific needs; it is an ideal tool for screening purposes, and could be used for the production of bacteriocins that are potentially deleterious to the producer. Quick evaluations of the effects of amino acid substitutions on bacteriocin activity can be made at the level of colonies of producer cells. Screening on the colony level implies that both changes in production and specific activity of bacteriocin molecules are assessed. A more detailed analysis of interesting mutants is required to ascertain the specific properties of the mutant bacteriocin. However, the variety of amino acids allowed at certain positions in the bacteriocin molecule can be quickly examined. This is illustrated for the cysteines at positions 24 and 44 of mature pediocin PA-1, where no active mutants were identified. Only base substitutions that leave the cysteines of the C24-C44 disulfide bridge intact yield active pediocin PA-1, indicating that it is very hard, if not impossible, to find single substitutes for either cysteine residue while still retaining bacteriocin activity. This result agrees with those of Miller et al. (30) and Fimland et al. (9) who describe that the C24-C44 disulfide bridge is required for (proper) pediocin PA-1 activity. It is, however, not unlikely that certain combinations of amino acids at positions 24 and 44 could result in active bacteriocins as many pediocin-like bacteriocins lack the second disulfide bridge (4, 11, 14, 16, 20, 21, 35, 40). Strains expressing pediocin PA-1 with amino acid substitutions at both C24 and C44 have not been isolated in our experiments but could be easily obtained by positive selection if the corresponding codons are simultaneously targeted for mutation.

Most amino acids can be inserted at position 34 of pediocin PA-1 with only a minor loss of wildtype activity. Replacing A34 with a branched-chain amino acid or an amino acid with a bulky aromatic group reduces bacteriocin activity. The complete loss of activity by the A34P substitution is probably due to a distorting effect that proline exerts on the putative C-terminal  $\alpha$ -helix. Alternatively, a lock in bacteriocin conformation due to the rigidity of the proline imino ring may be the reason for the loss of pediocin PA-1 activity. Equally, the loss of activity upon introduction of amino acids with larger or smaller side chains at position 34 may also be due to changes in the conformation or

flexibility of the backbone. This supposition is, to some extent, supported by the fact that larger side chains cause a larger reduction in activity, e.g. activity of PedA34V>PedA34I and of PedA34F>PedA34W/PedA34Y. Introducing a charged group, either negative or positive, at position 34 almost entirely abolishes bacteriocin activity. The mutant PedA34H has 80% of wildtype pediocin PA-1 activity, suggesting that histidine is not charged in its micro-environment and that, therefore, activity is hardly reduced. The low activity of PedA34D is in good agreement with the data from Miller et al. (30) who showed that an MBP-pediocin chimera with the same mutation has hardly any residual activity.

The loop between cysteines 9 and 14 of the first disulfide bridge in pediocin like-bacteriocins contains one dominant feature: the presence of one to three lysine residues. The glycine and serine residues at positions 10 and 13 in this loop are not very conserved. Thus, it is not surprising that pediocin PA-1 retains wildtype activity upon the introduction of an additional lysine (S13K) in this region of the molecule. Also, introduction of a glycine residue (S13G) is not a drastic change as a glycine is often found at various positions in this loop. Other changes (G10V, G10R, G10Y, S13T, S13A) are also not deleterious for pediocin PA-1 activity, indicating that there is quite a degree of tolerance for amino acid substitutions in this loop. Introduction of a proline (S13P), on the other hand, causes a drop in the activity, which could be caused by the side chain or by the rigidity introduced by the amino acid residue and suggests that the loop between cysteines 9 and 14 needs to adopt a specific conformation or flexibility. An NMR-study on leucococcin A has shown that this region is part of a  $\beta$ -turn (10).

The mutation analysis also demonstrates that, in addition to the cysteines at positions 24 and 44, certain other amino acids cannot be changed without strong effects on bacteriocin activity. At the position of glycine residue 40 large (G40Y) or charged (G40R, G40D) residues are detrimental to bacteriocin activity. This may be either due to distortion of the amphipathicity of the putative  $\alpha$ -helix (22) in this C-terminal region (G40R, G40D) or to conformational changes introduced by the bulkiness of the side chain (G40Y). Even side chains that introduce smaller changes (G40A, G40L, G40T) cause a reduction in bacteriocin activity. At position 18 a minor change from tryptophan to phenylalanine (W18F) already causes abolishment of bacteriocin activity. Thus, both a glycine residue at position 40 and a tryptophan residue at position 18 are essential for high pediocin PA-1 activity. The importance of the latter is supported by homology comparisons: in all pediocin-like bacteriocins except sakacin A this tryptophan is conserved. Sakacin A has no tryptophan at position 18 but has one at position 15 (16). Miller et al. (30) showed that a W18R substitution renders the bacteriocin almost inactive (less than 1% of wildtype activity).

The aspartic acid residue at position 17 is a quite well conserved residue in the pediocin-like bacteriocins: it is sometimes replaced by asparagine, a structurally

related amino acid. This would suggest that it is important for activity of the bacteriocin, a supposition supported by the fact that half of the mutants obtained for this residue are inactive. In some instances, however, not all activity is lost: cells producing pediocin PA-1 with the changes D17V, D17L or D17F lose up to about one third of the activity of native pediocin PA-1 against the indicators *Lb. saké*, *Lis. innocua* and *E. faecalis* 1 while they are completely inactive against *P. pentosaceus* PPE1.2 and *P. pentosaceus* FBB. The fact that all three mutant bacteriocins are completely inactive against *P. pentosaceus* PPE1.2 and *P. pentosaceus* FBB can be explained by assuming that the activity level has dropped to below the detection level. For the other indicators, however, the drop in activity is not the same for each indicator used. The activity against *E. faecalis* and *Lis. innocua*, with relative halo sizes of around 0.55, is clearly less affected than the activity against *Lb. saké* (relative halo sizes of approximately 0.29). Thus, Asp17 is more important for activity against *Lb. saké* than against *Lis. innocua* or *E. faecalis*, rendering the mutant bacteriocins more specific for *Lis. innocua* and *E. faecalis*, although they have lost some of their activity. This indicates that, unlike previously stated (8), not only the C-terminus is important in target cell specificity.

Creating novel bacteriocins with altered specificities is a challenging task. Introduced mutations will often result in a loss of activity. The system presented here allows to quickly screen for the variations that are allowed at a single amino acid position. This information, for any position in the molecule, can subsequently give clues as to the direction in which mutations can be made to improve known bacteriocins.

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## REFERENCES

1. Axelsson, L., T. Katla, M. Bjornslett, V. G. Eijsink, and A. Holck. 1998. A system for heterologous expression of bacteriocins in *Lactobacillus sake*. FEMS Microbiol.Lett. **168**:137-143.
2. Aymerich, T., H. Holo, L. S. Havarstein, M. Hugas, M. Garriga, and I. F. Nes. 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Appl Environ Microbiol **62**:1676-1682.
3. Barik, S. 1996. Site-directed mutagenesis in vitro by megaprimer PCR. Methods Mol Biol **57203-15**:203-215.
4. Bennik, M. H., B. Vanloo, R. Brasseur, L. G. Gorris, and E. J. Smid. 1998. A novel bacteriocin with a YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterization and interaction with target organisms. Biochim Biophys Acta **1373**:47-58.

5. Biet, F., J. M. Berjeaud, R. W. Worobo, Y. Cenatiempo, and C. Fremaux. 1998. Heterologous expression of the bacteriocin mesentericin Y105 using the dedicated transport system and the general secretion pathway. *Microbiology* **144**:2845-2854.
6. Chikindas, M. L., K. Venema, A. M. Ledebøer, G. Venema, and J. Kok. 1995. Expression of lactococcin A and pediocin PA-1 in heterologous hosts. *Lett Appl Microbiol* **21**:183-189.
7. Eijlsink, V. G., M. Skeie, P. H. Middelhoven, M. B. Brurberg, and I. F. Nes. 1998. Comparative studies of class IIa bacteriocins of lactic acid bacteria. *Appl Environ Microbiol* **64**:3275-3281.
8. Fimland, G., O. R. Blingsmo, K. Sletten, G. Jung, I. F. Nes, and J. Nissen-Meyer. 1996. New biologically active hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins: the C-terminal region is important for determining specificity. *Appl Environ Microbiol* **62**:3313-3318.
9. Fimland, G., L. Johnsen, L. Axelsson, M. B. Brurberg, I. F. Nes, V. G. Eijlsink, and J. Nissen-Meyer. 2000. A C-terminal disulfide bridge in pediocin-like bacteriocins renders bacteriocin activity less temperature dependent and is a major determinant of the antimicrobial spectrum. *J Bacteriol* **182**:2643-2648.
10. Fregeau-Gallagher, N. L., M. Sailer, W. P. Niemczura, T. T. Nakashima, M. E. Stiles, and J. C. Vederas. 1997. Three-dimensional structure of leucocin A in trifluoroethanol and dodecylphosphocholine micelles: spatial location of residues critical for biological activity in type IIa bacteriocins from lactic acid bacteria. *Biochemistry* **36**:15062-15072.
11. Fremaux, C., Y. Hechard, and Y. Cenatiempo. 1995. Mesentericin Y105 gene clusters in *Leuconostoc mesenteroides* Y105. *Microbiology* **141**:1637-1645.
12. Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other *lactic streptococci* after protoplast-induced curing. *J Bacteriol* **154**:1-9.
13. Geis, A., J. Singh, and M. Teuber. 1983. Potential of *Lactic Streptococci* to produce bacteriocin. *Appl Environ Microbiol* **45**:205-211.
14. Hastings, J. W., M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1991. Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. *J Bacteriol* **173**:7491-7500.
15. Henderson, J. T., A. L. Chopko, and P. D. van Wassenaar. 1992. Purification and primary structure of pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0. *Arch Biochem Biophys* **295**:5-12.
16. Holck, A., L. Axelsson, S. E. Birkeland, T. Aukrust, and H. Blom. 1992. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. *J Gen Microbiol* **138**:1-20.
17. Holo, H. and I. F. Nes. 1995. Transformation of *Lactococcus* by electroporation. *Methods Mol Biol* **47**:195-199.
18. Horn, N., M. I. Martinez, J. M. Martinez, P. E. Hernandez, M. J. Gasson, J. M. Rodriguez, and H. M. Dodd. 1999. Enhanced production of pediocin PA-1 and coproduction of nisin and pediocin PA-1 by *Lactococcus lactis*. *Appl. Environ. Microbiol.* **65**:4443-4450.
19. Jack, R. W., J. R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. *Microbiol Rev* **59**:171-200.
20. Jack, R. W., J. Wan, J. Gordon, K. Harmark, B. E. Davidson, A. J. Hillier, R. E. Wettenhall, M. W. Hickey, and M. J. Coventry. 1996. Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. *Appl Environ Microbiol* **62**:2897-2903.
21. Kalmokoff, M. L., S. K. Banerjee, T. Cyr, M. A. Hefford, and T. Gleeson. 2001. Identification of a New Plasmid-Encoded sec-Dependent Bacteriocin Produced by *Listeria innocua* 743. *Appl Environ Microbiol* **67**:4041-4047.
22. Kaur, K., L. C. Andrew, D. S. Wishart, and J. C. Vederas. 2004. Dynamic relationships among type IIa bacteriocins: temperature effects on antimicrobial activity and on structure of the C-terminal amphipathic alpha helix as a receptor-binding region. *Biochemistry* **43**:9009-9020.
23. Kemperman, R., A. Kuipers, H. Karsens, A. Nauta, O. Kuipers, and J. Kok. 2003. Identification and characterization of two novel clostridial bacteriocins, circularin A and closticin 574. *Appl. Environ. Microbiol.* **69**:1589-1597.
24. Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev* **12**:39-85.
25. Le Marrec, C., B. Hyronimus, P. Bressollier, B. Verneuil, and M. C. Urdaci. 2000. Biochemical and genetic characterization of coagulin, a new antilisterial bacteriocin in the pediocin family of bacteriocins, produced by *Bacillus coagulans* I-4. *Applied and Environmental Microbiology* **66**:5213-5220.
26. Leenhouts, K., A. Bolhuis, G. Venema, and J. Kok. 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. *Appl Microbiol Biotechnol* **49**:417-423.

27. **Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledebøer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. Zoetmulder, and P. A. Vandenberg.** 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, and bacteriocin from *Pediococcus acidilactici* PAC1.0. *Appl. Environ. Microbiol.* **58**:2360-2367.
28. **Metivier, A., M. F. Pilet, X. Dousset, O. Sorokine, P. Anglade, M. Zagorec, J. C. Piard, D. Marion, Y. Cenatiempo, and C. Fremaux.** 1998. Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization. *Microbiology* **144**:2837-2844.
29. **Miller, K. W., R. Schamber, Y. Chen, and B. Ray.** 1998. Production of active chimeric pediocin AcH in *Escherichia coli* in the absence of processing and secretion genes from the *Pediococcus* pap operon. *Appl Environ Microbiol* **64**:14-20.
30. **Miller, K. W., R. Schamber, O. Osmanagaoglu, and B. Ray.** 1998. Isolation and characterization of pediocin AcH chimeric protein mutants with altered bactericidal activity. *Appl Environ Microbiol* **64**:1997-2005.
31. **Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek* **70**:113-128.
32. **Nes, I. F. and H. Holo.** 2000. Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* **55**:50-61.
33. **Nissen-Meyer, J. and I. F. Nes.** 1997. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis, and mechanism of action. *Arch. Microbiol.* **167**:67-77.
34. **Pucci, M. J., E. R. Vedamuthu, B. S. Kunka, and P. A. Vandenberg.** 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. *Appl Environ Microbiol* **54**:2349-2353.
35. **Quadri, L. E., M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles.** 1994. Chemical and genetic characterization of bacteriocins produced by *Carnobacterium piscicola* LV17B. *J Biol Chem* **269**:12204-12211.
36. **Rodriguez, J. M., M. I. Martinez, N. Horn, and H. M. Dodd.** 2003. Heterologous production of bacteriocins by lactic acid bacteria. *Int. J. Food Microbiol.* **80**:101-116.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press.
38. **Sanders, J. W., G. Venema, and J. Kok.** 1997. A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis*. *Appl Environ Microbiol* **63**:4877-4882.
39. **Sanders, J. W., G. Venema, J. Kok, and K. Leenhouts.** 1998. Identification of a sodium chloride-regulated promoter in *Lactococcus lactis* by single-copy chromosomal fusion with a reporter gene. *Mol. Gen. Genet.* **257**:681-685.
40. **Tichaczek, P. S., R. F. Vogel, and W. P. Hammes.** 1994. Cloning and sequencing of *sakP* encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. *Microbiology* **140**:361-367.
41. **van de Guchte, M., J. M. van der Vossen, J. Kok, and G. Venema.** 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. *Appl Environ Microbiol* **55**:224-228.
42. **Venema, K., J. Kok, J. D. Marugg, M. Y. Toonen, A. M. Ledebøer, G. Venema, and M. L. Chikindas.** 1995. Functional analysis of the pediocin operon of *Pediococcus acidilactici* PAC1.0: PedB is the immunity protein and PedD is the precursor processing enzyme. *Mol Microbiol* **17**:515-522.



